Kidney International, Vol. 44 (1993), pp. 764–773

Renin stimulating properties of parathyroid hormone-related peptide in the isolated perfused rat kidney

CHRISTIAN SAUSSINE, THIERRY MASSFELDER, FREDERIQUE PARNIN, CLEMENT JUDES, UMBERTO SIMEONI, and JEAN-JACQUES HELWIG

Laboratoire de Physiologie Cellulaire Rénale, Faculté de Médecine, Université Louis Pasteur, Strasbourg, France

Renin stimulating properties of parathyroid hormone-related peptide in the isolated perfused rat kidney. Previous studies showed that PTHrP exhibits renal vasodilating, arteriolar cAMP stimulating and receptor binding properties. The present experiments were designed to study whether PTHrP may influence renin secretion. Rat kidneys were isolated and single-pass perfused at constant flow and stabilized pressure. Exposures to PTHrP or PTH stimulated a dose-dependent renin release reaching similar V_{max}. The affinity (0.1 nM) and threshold concentration (0.01 nM) for PTHrP were about 10 times lower than for PTH. Compared to 10 μM isoproterenol, the maximum renin responses to PTHrP were similar but of shorter duration. The PTHrP dose-response curve was not affected by 10 μM indomethacin. Administered simultaneously, PTHrP and PTH displayed no additive effects. PTHrP-induced renin release as well as the role of extracellular calcium were further studied in nonfiltering kidneys, which were perfused at a constant flow and stable pressure in a closed circuit. Basal renin release was inversely related with perfusate calcium and was depressed by the calcium ionophore BAY-K8644. PTHrP (100 nM) induced a 1.6-fold increase of basal renin release in normocalcic perfusate. Removing calcium abolished renin responses. PTHrP reversed the inhibiting effects of hypercalcic media or BAY-K8644 on basal renin release. The results support calcium-mediated renin stimulating properties for PTHrP, via PTH receptors, independently from baroreceptors, macula densa and prostaglandins.

Parathyroid hormone-related peptide (PTHrP) has been shown to be the primary tumor-derived hypercalcemic factor involved in the pathogenesis of tumor-associated hypercalcemia [reviewed in 1–4]. PTH and PTHrP are expressed by different genes and are immunologically distinct but have limited homology in the amino-terminus portion. PTH and PTHrP share the same target tissues and possess common physiological properties, including stimulation of bone resorption and elevation of blood calcium concentration.

Like PTH, PTHrP interacts with cardiovascular tissues. When administered systematically, PTHrP reduces blood pressure, increases blood flow and/or decreases vascular resistance in the kidney, muscle and skin [5, 6]. *In vitro*, PTHrP vasorelaxes isolated segments of the rabbit renal artery [7] and helical strips of the rat aorta [5]. Furthermore, an increasing number of normal tissues [8–10], including extravascular [10–14] and vascular smooth muscles [8–10, 14, 15], express mRNA for PTHrP or PTHrP itself. Thus, in contrast to PTH, which is exclusively secreted by the parathyroid glands, PTHrP could serve as a local modulator of regional microcirculation.

We recently studied whether this peptide may be active on the intrarenal vascular bed. In rabbit renal microvessel preparations enriched in glomerular arterioles, both PTHrP and PTH were found to bind to common and saturable receptors. The affinity and number of PTHrP receptors in the microvessels were similar to those of renal tubules [16]. Also, PTHrP induces a concentration-related relaxation of the rat isolated perfused kidney (IPK) preconstricted with the prostaglandin $PGF_{2\alpha}$ [17]. As PTHrP stimulates the adenylyl cyclase activity in the isolated renal microvessels, the cAMP system seems to be involved in the renal vascular action of PTHrP [17]. These influences of PTHrP on the renal vascular bed cause questions of whether PTHrP may also influence other functions of the renal vascular smooth muscle cells, such as renin secretion, like many other renal vasodilators. Moreover, factors which stimulate renin release appear to do so mainly by lowering cytosolic calcium and/or increasing cellular cAMP [18, 19]. In support of such a hypothesis, we recently showed that rat(r)PTH-(1-34) not only stimulates adenylyl cyclase in renal cortex microvessels or vasodilates the rat IPK [17, 20], but also stimulates renin release, in a calcium-dependent manner from rat IPK, isolated glomeruli and dispersed cortical cells [21, 22].

The aims of the present study were to explore the concentration-related effect of PTHrP on renin release, to compare the renin-stimulating potency of PTHrP to that of PTH, and to ask whether the vasodilator products of cyclooxygenase activity may account for the renin release in response to PTHrP and whether PTHrP and PTH stimulate renin secretion via common action sites. Finally, we also queried if the stimulating effect of PTHrP on renin release is dependent on extracellular calcium. We chose to conduct these studies in an isolated rat kidney model, perfused at constant flow and stable pressure to obviate the involvement of the baroreceptors in the renin release responses.

Methods

Drugs

Drugs were obtained from the following sources: human (h)PTHrP-(1-34) and rat(r) PTH-(1-34) (Bachem Feinchemikalien AG, Bubendorf, Switzerland; Cambridge Research Biochemicals Ltd., Northwich, UK; Neosystem Laboratories,

Received for publication February 22, 1993
and in revised form April 23, 1993
Accepted for publication May 27, 1993

Strasbourg, France); the peptides were dissolved in 1 mM HCl and 1 mg/ml BSA at a concentration of 0.25 mM and stored in 25 μl portions at −70°C; BSA, fraction V (Euromedex, Schiltigheim, France), was dialyzed overnight against Krebs-Henseleit buffer; phenylmethylsulfonyl fluoride, 8-hydroxyquinoline, indomethacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid) and (−)-isoproterenol hydrochloride (Sigma Chemical Co., St. Louis, Missouri, USA); (3-[^{125}I]-iodotyrosyl-4)angiotensin I (Amersham, Buckinghamshire, UK); angiotensin I (Ang I, Peninsula Labs, Belmont, California, USA); rabbit anti-Ang I (Calbiochem, La Jolla, California, USA); sodium pentobarbital (Clin Midy, St. Jean de la Ruelle, France).

Studies in the single-pass open circuit rat IPK

Kidney preparation. Male Wistar rats (170 to 220 g) were allowed free access to standard food and water. The animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (45 mg/kg). After isolation of the kidney and ligation of the suprarenal aorta, perfusion through the superior mesenteric artery was started without ischemia according to the basic description of Schurek, Brecht and Hierholzer [23] with some modifications. After complete separation from surrounding connective tissue, the kidney was taken out from animal body and put on a thermostatically-controlled holder, and perfused in a single-pass open circuit. The perfusate was continuously gassed with 95% O_2 and 5% CO_2 and warmed at 37°C. The perfusion flows, expressed as ml/min/g of controlateral kidney, were adjusted to achieve a perfusion pressure of about 80 mm Hg during the first 30 minutes and remained constant thereafter. At least 110 minutes were allowed for equilibration, after which the kidneys were perfused from secondary reservoirs with perfusates containing the desired final concentrations of drugs (peptides or isoproterenol), or from a main reservoir with perfusate containing no drug. In each of the six studies to be described, perfusate collections for renin activity determination were started at the end of the equilibration period up to a maximum of 200 minutes. Samples of 1 ml from each collected fraction were cooled at −20°C before assayed for renin activity. Perfusion pressures of the infrarenal aorta were monitored continuously with a pressure transducer (Statham P23Db; Statham Laboratories Inc., Hato Rey, Puerto Rico). Kidney preparations in which the perfusion flows reached about 80 mm Hg were lower than 10 ml/min/g or those in which the overall pressure variations were higher than 12 mm Hg during the 110 to 200 minute experimental period were systematically rejected.

Experimental protocol. Six independent groups were studied using the single-pass open circuit IPK. Group 1 (N = 5); time controlled kidneys received no treatment. Perfusate was collected over six minute periods between 110 and 200 minutes. Group 2 (N = 5; PTHrP dose response) kidneys were treated with increasing concentrations of hPTHrP-(1-34) (0.01 nM to 100 nM) every 18 minutes (at 110, 128, 146, 164 and 182 min) for two minutes. Perfusate was collected over consecutive six-minute periods between 110 and 200 minutes. At the end of each 18 minute period, or before each PTHrP treatment, an additional 10 second perfusate collection was performed. These 10 second fractions served to check that before each PTHrP treatment, the renin release rate returned to a value similar to that measured during the corresponding six minute period in time control IPK. These 10 second fractions were designated as "basal renin release checking points." The group 3 (N = 6; PTH dose response) group was identical to group 2 except that hPTHrP-(1-34) was replaced by rPTH-(1-34). Group 4 (N = 4) tested the effect of indomethacin on the PTHrP dose response. This group is completely analogous to group 2 except that 10 μM indomethacin was present in the perfusate. In group 5 (N = 4); comparative effects of maximal concentrations of rPTH-(1-34), hPTHrP-(1-34) and isoproterenol) kidneys were treated with 100 nM hPTHrP, 100 nM rPTH and 10 μM isoproterenol at 128, 146 and 164 minutes, respectively, during two minutes. Six-minute perfusate collections were performed as described for group 2. Ten second perfusate collections (basal renin release checking points) were performed before each treatment and at 200 minutes. Group 6 (N = 6; additivity of PTHrP- and PTH-induced renin release) kidneys were successively treated for two minutes with 100 nM hPTHrP, 100 nM rPTH and simultaneously with both peptides. An injection cycle time of 18 minutes was employed starting at the 128th minute and perfusate collected over six minute periods as before, but the 10 second perfusate collections (basal renin release checking points) were not performed in this final group. PTHrP and PTH were administered in the first and second position alternatively to rule out an interacting effect on their renin stimulatory responses.

Perfusion media for the open circuit IPK. The basic perfusion solution was a Krebs-Henseleit bicarbonate buffer, pH 7.4, containing 30 mM sodium lactate and 3% gelatin as the oncotic agent. Ionized calcium concentrations (1 mM) in the perfusate and pH were adjusted with a Ca^{2+}-pH analyzer (Ciba Corning Diagnostic Co., Medfield, USA).

The recirculating closed circuit IPK

After complete separation from surrounding animal body as described above, the kidney was perfused in a closed circuit by recovering the effluent flowing from the vena cava directly into a recirculating reservoir. The recirculating perfusion volume was 50 ml in each experiment, and the perfusate was gassed continuously with 95% O_2 and 5% CO_2. The kidney preparations were rendered nonfiltering by the use of perfusate with high oncotic pressure (100 mg/ml BSA), a low perfusion pressure (70 mm Hg), and by ligation of the ureter [24]. Perfusion flows, expressed as ml/min/g of controlateral kidney, were adjusted to achieve a perfusion pressure of 70 mm Hg during the first 10 to 15 minutes and remained constant for the rest of the experiment which lasted 90 minutes. Perfusion pressures of the infrarenal aorta were monitored as described above. Flow and pressure criteria for exclusion of kidney preparations were similar to those described above for the open circuit.

Experimental protocol. Four independent groups, differing by the ionized calcium content of the perfusate or the presence of calcium effectors, were studied using the recirculating closed circuit IPK. Each group was divided into separate time-control (CTL) and PTHrP-treated kidney preparations. The ionized calcium concentration in the perfusate was adjusted to either 1 mM (group A) or 2 mM (group B). In a third group, BAY-K8644, a calcium channel agonist, was added into the recirculating reservoir after 10 to 15 minutes of perfusion (group C). The last group was perfused with calcium-free medium containing EGTA as a calcium-chelating agent (group D). In all of the

PTHrP-treated kidney preparations, hPTHrP-(1-34) was added in the recirculating reservoir at 38 minutes. In the four groups, the time course of the PTHrP-treated preparation was compared to the corresponding time-control preparation. The time intervals taken for the time course and the sampling points were identical, whatever the kidney groups. Perfusate samples of 50 μl were collected at five minute intervals, beginning at the end of the equilibration period (35 min) up to 90 minutes. The samples were cooled at −20°C and diluted 25 times before assayed for renin activity.

Perfusion media for the closed circuit. The basic composition of the perfusion solution was, in mM: glucose, 5; urea, 6; Na oxaloacetate, 1; Na lactate, 5; Na pyruvate, 2; Na glutamate, 5; L-methionine, 0.5; L-alanine, 2; glycine, 2; L-serine, 2; L-proline, 2; L-isoleucine, 1 and L-aspartic acid, 3 in Krebs-Henseleit Bicarbonate buffer containing 10% fraction V BSA. Ionized calcium and pH were adjusted as described above for the open circuit perfusion medium. BAY-K8644 was dissolved in ethanol at a concentration of 5 mM and then further diluted in perfusion medium to 5 μM, then 0.2 ml of this solution was added to the 50 ml circulating medium to achieve a final concentration of 20 nM. All experiments in which BAY-K8644 was used were performed in the dark under monochromatic light due to the light sensitivity of this compound. PTHrP-treated kidneys received 25 μl of 250 μM hPTHrP-(1-34) in 1 mM HCl, 0.1% BSA to achieve a concentration of 125 nM in the perfusate volume.

Determination of renin activity

Twenty microliters of the various samples of perfusate were incubated in duplicate with an excess of partially purified angiotensinogen from rats bilaterally nephrectomized 48 hours before, according to the method of Itoh, Carretero and Murray [25]: 5 mM 8-hydroxyquinoline (converting enzyme inhibitor) and 66.6 mM phosphate buffer, pH 6.5 containing 13.5 mM EDTA in a total volume of 100 μl for one hour at 37°C. The amount of substrate in each tube ranged from 200 to 400 ng Ang I equivalent, and the amount of generated Ang I never exceeded 5 ng per tube. The reaction was stopped by boiling for three minutes and the addition of 0.2 ml of 66.6 mM phosphate buffer, pH 7.5 containing 1 mg/ml Na azide and 1 mg/ml BSA. After centrifugation for 15 minutes at 2500 × g, Ang I was measured in 0.1 ml supernatant by RIA, according to the method of Fyhrquist et al. [26]. The various substrate preparations had neither measurable Ang I nor renin activity. Angiotensinase activity was also absent from substrate preparations or samples since the recovery of added Ang I was near 100%.

Units, calculations and statistics

The rate of renin release was expressed as ng Ang I generated in one hour incubation per minute of perfusion and normalized per g of kidney weight, taking the weight of the controlateral kidney as the weight of the perfused kidney (ng Ang I/hr × min^{-1} × g^{-1} or Ang I units).

Open circuit IPK. In groups 2 to 6, the mean renin release rate in the six minute fractions are presented as net increase (mean ± SEM) by subtracting the corresponding mean renin release rates obtained from group 1 (time control). The PTH and PTHrP dose response curves were built by calculating the overall net renin release rates during the 18 minute periods following the administrations of increasing concentrations of peptide.

Table 1. Flows and pressures in single-pass IPK

IPK group	KW *g*	P *mm Hg*	ΔP_{max} *mm Hg*	Flow *ml/min/g*	RVR *mm Hg/ flow*
Group 1	0.75 ± 0.01	70.6 ± 2.6	8.6 ± 2.4	11.3 ± 0.4	6.3 ± 0.3
Group 2	0.71 ± 0.04	76.1 ± 6.4	10.1 ± 2.6	11.8 ± 1.1	6.8 ± 1.1
Group 3	0.69 ± 0.04	76.4 ± 3.1	9.1 ± 1.9	11.7 ± 0.5	6.6 ± 0.5
Group 4	0.76 ± 0.08	80.6 ± 3.0	7.0 ± 2.2	11.5 ± 0.9	7.2 ± 0.7
Group 5	0.75 ± 0.01	86.0 ± 2.8	5.8 ± 1.8	10.6 ± 0.2	8.1 ± 0.3
Group 6	0.71 ± 0.05	81.5 ± 2.8	5.8 ± 2.7	12.0 ± 0.8	7.2 ± 0.4

Abbreviations are: kidney weight: KW; mean pressure (P), overall pressure variation (ΔP_{max}) and mean renal vascular resistance (RVR) in the course of the experimental period. For each parameter, there was no significant difference between the 6 groups ($P > 0.100$) in the analysis of variance for single factor experiments.

Closed circuit IPK. As the perfusate recirculates in groups A to D, renin activity measured at any time represents cumulative activity in Ang I units. For purposes of comparisons between time-control and PTHrP-treated preparations inside each IPK group, the renin release time courses are presented as the net renin activity increase from the end of the initial equilibration period by subtracting the renin activity present at 35 minutes from all subsequent values. The rates of renin release were determined over the following time periods: 0 to 35 minutes, 35 to 50 minutes, 50 to 65 minutes, 65 to 80 minutes, and 80 to 90 minutes. The rate of renin release was calculated as follows: renin release rate = (Ang I units)t_2 − (Ang I units)$t_1/t_2 - t_1$ (ng Ang I units/min), where t_1 and t_2 represent the beginning and the end of the various period intervals.

As a general rule, data points obtained under the different experimental conditions were subjected to analysis of variance for factorial experiments [27]. When significant differences were found, a Duncan multiple range and multiple F-test [28] was applied to compare point by point and to identify significant differences between individual means. A 95% confidence level ($P < 0.05$) was considered to reflect statistical differences. Deviations from mean values were expressed as the SEM.

Results

Renin stimulating properties of PTHrP and PTH in the single-pass IPK

Flows and pressures. During the experimental period (110 to 200 min) there were no significant differences between the six groups with respect to kidney weights, mean perfusion pressures or perfusate flow rates as well as the resulting renal vascular resistances (Table 1). Since perfusate flow was adjusted during the first 30 minutes and kept constant throughout the remainder of the experiment, the perfusion pressures were continuously recorded during the experimental period. As shown in Table 1, the present methodology maintained the overall variations of pressures within a narrow range during the 90 minute experimental period.

Comparison of renin release time courses from time-control kidneys and kidneys exposed to graded concentrations of hPTHrP-(1-34) and rPTH-(1-34) (groups 1 to 3). In time-control IPK, the basal renin release rate decreased slowly and linearly

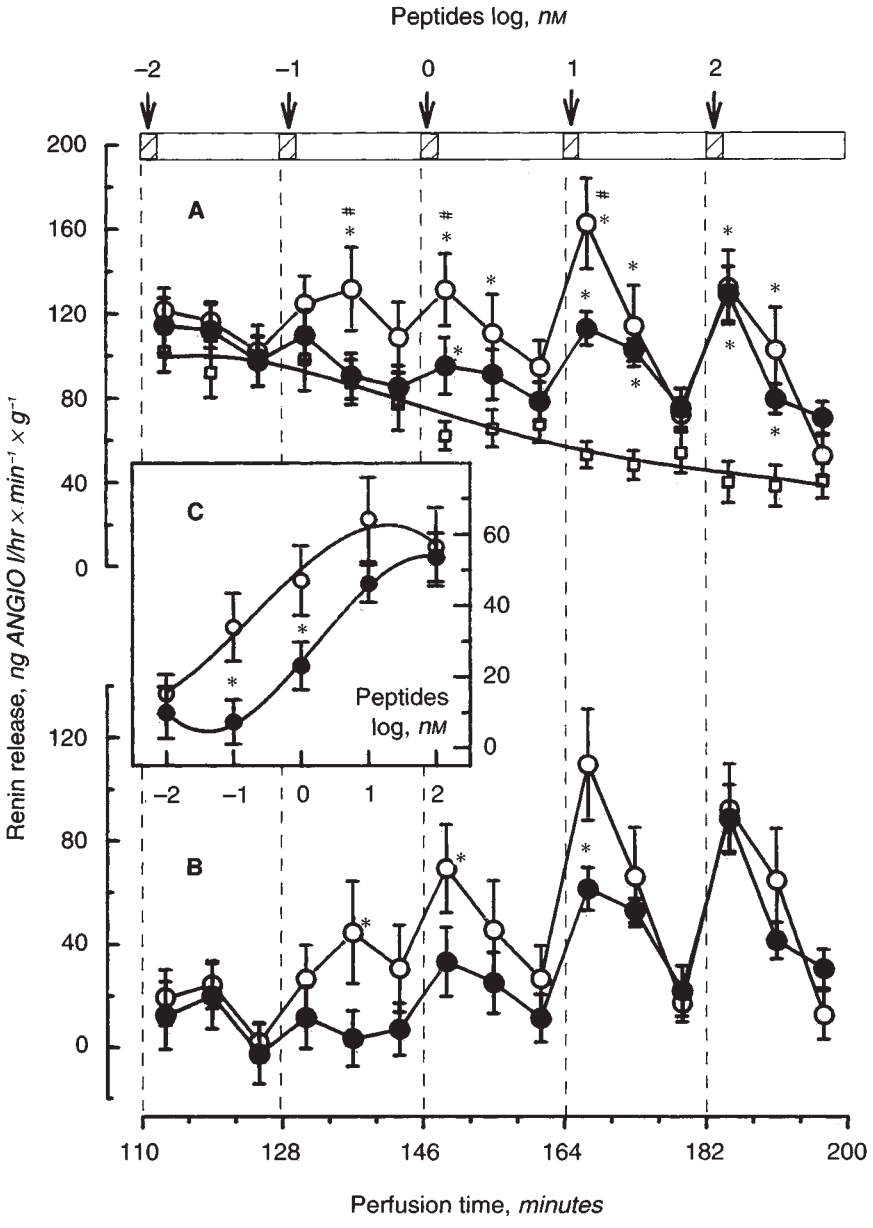

Fig. 1. *Renin release from single-pass IPK.* **A.** Comparison of renin release time courses from time-control kidneys (group 1; $N = 5$) and kidneys exposed to graded concentrations of hPTHrP-(1-34) (group 2; $N = 5$) or rPTH-(1-34) (group 3; $N = 6$). The increasing concentrations of peptide were added for two minutes every 18 minutes as indicated by the striped and open areas of the top horizontal bar. Data points represent the average of renin release rate during consecutive six minute periods of perfusate collection. Statistical significance ($P < 0.05$) for a given perfusion period was determined by comparing time control IPK to either PTHrP- or PTH-treated group (*) or by comparing PTHrP-treated group to PTH-treated group (#) as described in **Methods**. **B.** Comparison of the net renin release responses to graded concentrations of PTHrP (group 2) and PTH (group 3). Data points represent the average of *net* renin release rate over six minute periods of perfusate collection, by subtracting the basal renin release rate obtained from time control IPK (group 1) during the corresponding period. Statistical significance ($P < 0.05$) for a given perfusion period was determined by comparing PTHrP-treated group to PTH-treated group (*) as described in **Methods**. **C.** Comparison of the dose response curves of PTHrP- and PTH-stimulated renin release as calculated from A and B. The data points represent the average of *net* renin release rate calculated over the 18 minute period following peptide injections. Asterisks indicate significant difference ($P < 0.05$) for a given peptide concentration.

from about 100 Ang I units to 40 Ang I units over the whole experimental period (Fig. 1A). The fact that renin release steady-state was not reached after 200 minutes of perfusion prompted us to compare the time course of renin release rate between control and peptide- or isoproterenol-treated IPK groups over the 110 to 200 minute experimental period, during which the perfusion pressure changes were limited.

PTHrP administration during two minutes every 18 minutes (group 2) resulted in a concentration-dependent increase in the mean renin release rate in these single-pass, stabilized pressure perfused rat kidneys (Fig. 1A). A point-by-point comparison (analysis of variance) with group 1 showed that the threshold concentration of PTHrP that induced a significant increase of the rate of renin release was 0.1 nM, and maximal effect was reached with 10 nM of PTHrP. Administration of PTH in the same molar concentrations and under identical experimental conditions also resulted in a concentration-related increase of the mean renin release rate (group 3); however, the threshold concentration of PTH was only 1 nM, while maximal effect was reached with 100 nM of PTH (Fig. 1A).

For purposes of direct comparisons between PTHrP- and PTH-induced responses, the renin release time courses plotted in Figure 1B have been presented as net increases by subtracting the renin activity present in time control kidneys during the same six minute perfusion period. A point-by-point comparison (analysis of variance) showed that the extent of the responses to 0.1, 1 and 10 nM PTHrP was significantly higher than those responses to PTH. In order to ensure that in these experiments the renin secretory rates actually returned to baseline values, renin activity released during the last 10 seconds before each PTHrP or PTH addition was compared to that measured during the corresponding six minute period in time-control IPK. As

Table 2. Basal renin release checking points in groups 2 to 5 as compared to time-control group 1 in single-pass IPK

Group 1 (Time-control)	Group 2	Group 3	Group 4	Group 5
104 to 110 min			109^{50} to 110 min	
118 ± 16	126 ± 21	110 ± 11	84 ± 18	ND
122 to 128 min			127^{50} to 128 min	
100 ± 15	106 ± 14	96 ± 10	104 ± 26	85 ± 14
140 to 146 min			145^{50} to 146 min	
78 ± 14	90 ± 15	80 ± 10	78 ± 22	77 ± 6
158 to 164 min			163^{50} to 164 min	
67 ± 9	69 ± 11	69 ± 8	65 ± 11	68 ± 4
176 to 182 min			181^{50} to 182 min	
49 ± 10	53 ± 8	66 ± 9	60 ± 13	ND
194 to 200 min			199^{50} to 200 min	
40 ± 8	49 ± 10	58 ± 8	60 ± 12	59 ± 4

In the analysis of variance for single factor experiments, the renin release rates measured during the 10 second periods (called basal renin release checking points) in groups 2 to 5, were not significantly different ($P > 0.1$) from those measured during the corresponding 6 minute periods in group 1 (time-control).

shown in Table 2, the renin release rates measured during these so-called basal renin release checking points in group 2 and 3 were not significantly different from those measured over the corresponding six minute periods in group 1. This observation indicated that renin release returned to baseline level before each peptide administration.

For purposes of direct comparison of PTHrP and PTH concentration-related curves, the results of Figure 1C have been presented as the mean secretory rates over the 18 minute periods which followed each peptide administration. In this comparison, the concentration of rat PTH producing half-maximal stimulation was 12 times higher (EC_{50} of 1.2 nM) than that of human PTHrP (EC_{50} of 0.1 nM).

Effect of indomethacin on the PTHrP dose response (group 4). Group 4 was conducted to explore the role of cyclooxygenase activity products in the responses to PTHrP observed in group 2. As shown in Figure 2, during perfusion in the presence of 0.1 μM of indomethacin (group 4), renin release responses were unchanged over an identical time sequence as in the previous study (group 2). Again, the renin release rates measured during the basal renin release checking points in group 4 were not significantly different from those measured over the corresponding six minute periods in group 1 (Table 2).

Comparative effects of maximal concentrations of rPTH-(1-34), hPTHrP-(1-34) and isoproterenol (group 5). Group 5 was conducted to compare the responses to the highest concentrations of PTHrP and PTH to those induced by a known stimulator of renin release. Isoproterenol at 0.1 μM was used (Fig. 3). The administration of 100 nM PTHrP for two minutes was followed by 100 nM PTH 18 minutes later, and resulted in renin release increases similar to those observed in groups 2 and 3 (Fig. 1B). However, if renin release rate returned to control values within 18 minutes after PTHrP or PTH administration (see group 5 in Table 2), the net renin released in response to isoproterenol lasted at least 36 minutes. Thus, the mean secretory rates in response to isoproterenol were 3.2 ± 0.6 and 3.4 ± 0.5 times higher than those to PTHrP and PTH, respectively.

Additivity of PTHrP- and PTH-induced renin release (group 6). The goal of the group 6 was to test the hypothesis that PTHrP and PTH stimulate renin release via common action sites. For that purpose, we determined whether maximal concentrations of PTHrP and PTH, administered together, resulted in additive renin release responses or not, as compared to those produced by each peptide alone. Expressed as mean secretory rates over 18 minute periods, PTHrP and PTH (100 nM) administered separately, increased renin release by 63 ± 6 and 70 ± 7 Ang I units, respectively, values similar to those shown in Figure 1C (56 ± 11 and 54 ± 7 Ang I units, respectively). In addition, the renin release responses to either PTHrP or PTH were the same whether PTHrP was administered before PTH or inversely. PTHrP and PTH (100 nM) administered together increased renin release by 64 ± 6 Ang I units, a value very similar to that obtained in response to each peptide alone.

Calcium dependency of renin stimulation by PTHrP in the recirculating IPK

The second set of experiments was conducted to test the hypothesis that the PTHrP-mediated renin release is dependent on extracellular calcium. For that purpose we used a nonfiltering rat IPK in order to minimize the interferences mediated by macula densa mechanisms, with the responses of basal and calcium-modulated renin release to PTHrP. It has previously been shown that the use of low perfusion pressure together with high perfusate oncotic pressure abolished the glomerular filtration [24]. Therefore, perfusate containing 10% BSA was used. Because the single-pass IPK requires large volumes of perfusate, and due to the cost of BSA, recirculating IPKs were employed.

Flows and pressures. The experimental protocols used in this series of experiments are summarized in Table 3, and the perfusion flows are shown in Table 4. The mean perfusion flow applied to reach 70 mm Hg in the four groups of IPK was 16.2 ± 0.4 ml/min × g, and it was not significantly different between time-control and PTHrP-treated IPK. As perfusate flows were adjusted during the first 10 to 15 minutes and kept constant throughout the remainder of the experiment, the perfusion pressures were recorded during the experimental period. As shown in Figure 4, the perfusion pressures did not change immediately after PTHrP injection. On the other hand, the pressures slowly increased, especially in time-control IPK. These increases were a little more obvious in time-control IPK from the hypercalcic group (group B) where the perfusion pressure reached 76.3 ± 0.8 mm Hg at 90 minutes. PTHrP seemed to protect the IPK from this small constriction in hypercalcic (group B) and EGTA (group D) groups. Whatever, the pressures of the IPK used for the present study may be considered to have been maintained within a narrow range between 35 and 90 minutes of perfusion, as the overall amplitude in pressure variation did not exceed 6 mm Hg.

Calcium modulation of basal renin release. As a rule, the renin activity in the recirculating perfusate rose spontaneously with time in control preparations inside each group (Fig. 5). In normocalcic medium (Fig. 5A) renin activity reached a maximal level at about 65 minutes and tended to stop thereafter. In preliminary experiments these observations prompted us to inject PTHrP after 70 minutes of equilibration; however, the rise in perfusion pressure became too great in a number of IPK after 90 to 100 minutes, especially those perfused with hypercalcic medium. Therefore, the time course of renin release

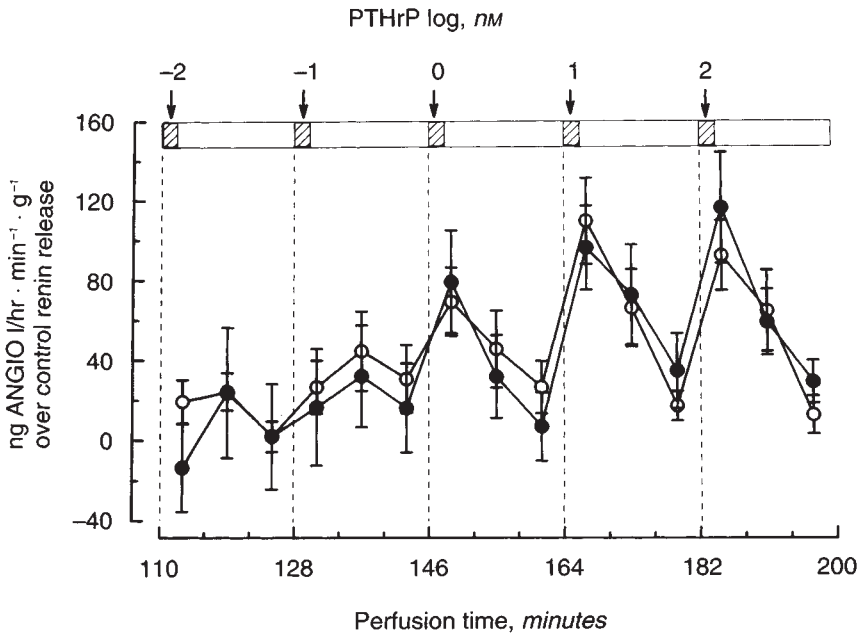

Fig. 2. *Effect of indomethacin on PTHrP-induced renin release in single-pass IPK.* Kidneys were perfused with increasing concentrations of PTHrP for two minutes every 18 minutes as indicated by the striped and open areas of the top horizontal bar, either in absence (○; group 2; $N = 5$) or presence of 0.1 μM indomethacin (●; group 4; $N = 4$). Data points represent the average of *net* renin release rate over six minute periods, by subtracting the basal renin release rate obtained from time control IPK shown in Figure 1A during the corresponding period of perfusate collection.

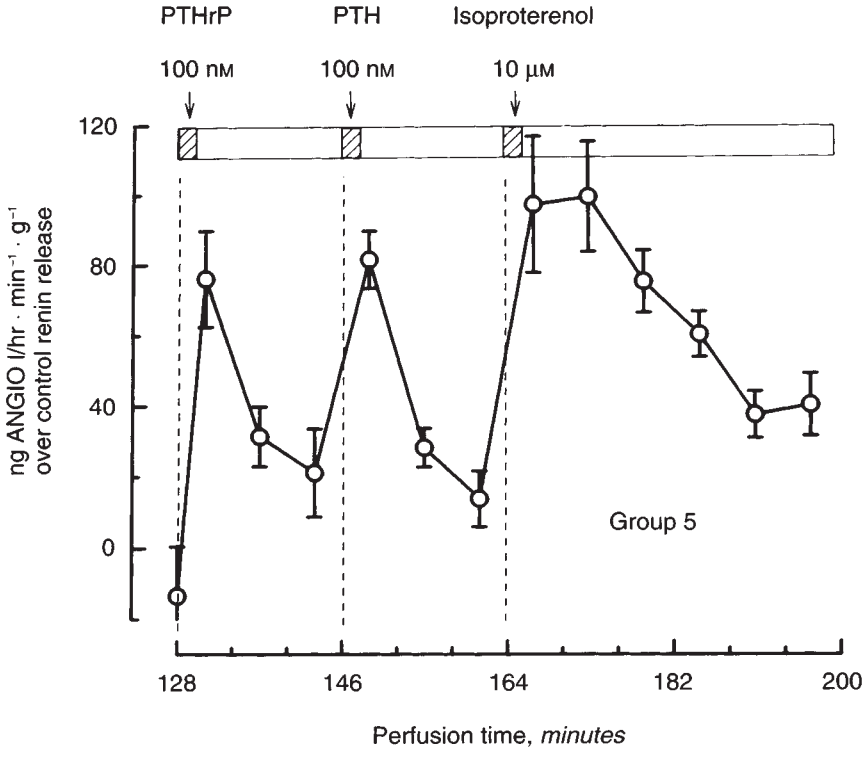

Fig. 3. *Comparison of net renin release responses to maximal concentrations of PTHrP, PTH and Isoproterenol (group 5;* N *= 4) in single-pass IPK.* Peptides and isoproterenol were added for two minutes every 18 minutes, as indicated by the striped and open areas of the top horizontal bar. Data points represent the average of *net* renin release rate over a six minute period, by subtracting the basal renin release rate obtained from time control IPK shown in Figure 1A during the corresponding period of perfusate collection.

between time control and PTHrP-treated preparations was compared over a 35 to 90 minute period, during which the perfusion pressure variations were limited. The comparison between the time course of spontaneous basal renin release (time control) indicated that the extent of renin activity which accumulated over the 35 to 90 minute time interval (Fig. 5) or the renin secretory rates over the 35 to 50, 50 to 65, 65 to 80 and 80 to 90 minute periods (Fig. 6) varied significantly with perfusate ionized calcium concentration or calcium effector addition. Increasing ionized calcium to 2 mM (panel B) or adding BAY-K8644 (panel C) decreased the amount of basal renin released or the mean secretory rates as compared to normocalcic time-control IPK (panel A). On the other hand, the use of calcium-free perfusate (panel D), though not obvious,

Table 3. Experimental conditions applied to the four groups of recirculating IPK

Designation of IPK group	N	Ionized Ca	Ca effector	hPTHrP-(1-34)[a]
Group A				
"normocalcic," CTL	6	1 mM	none	none
"normocalcic," PTHrP	6	1 mM	none	125 nM
Group B				
"hypercalcic," CTL	6	2 mM	none	none
"hypercalcic," PTHrP	6	2 mM	none	125 nM
Group C				
"BAY-K8644," CTL	4	1 mM	20 nM BAY-K8644[b]	none
"BAY-K8644," PTHrP	4	1 mM	20 nM BAY-K8644	125 nM
Group D				
"EGTA," CTL	6	<0.01 mM	2 mM EGTA[c]	none
"EGTA," PTHrP	6	<0.01 mM	2 mM EGTA	125 nM

N is the number of independent IPK preparations. Note that in each experimental group, the *N* time-control (CTL) IPK and the *N* PTHrP-treated IPK are separate kidney preparations.

[a] hPTHrP-(1-34) was administered at 38 minutes.

[b] BAY-K8644 was administered between 10 and 15 minutes.

[c] EGTA was included during perfusate preparation.

Table 4. Perfusion flows in the four groups of recirculating IPK

IPK Groups	Time-control	PTHrP-treated
Group A	19.0 ± 1.9	16.8 ± 1.5
Group B	15.7 ± 1.1	16.3 ± 1.1
Group C	16.3 ± 1.8	15.2 ± 1.8
Group D	15.5 ± 1.1	14.8 ± 0.4

Flows applied to reach about 70 mm Hg are expressed as ml/min/g kidney weight. A, B, C and D refer to the experimental conditions described in Table 3.

tended to increase the spontaneous basal renin release especially during the 50 to 90 minute period as compared to normocalcic time-control IPK (panel A).

Calcium modulation of PTHrP-stimulated renin release. With normocalcic medium, renin release in the presence of PTHrP rose for up to 80 minutes, reaching a maximal value of 935 ± 106 Ang I units, which was 1.6 times higher than in the control where a near maximal value was reached at 65 minutes. PTHrP increased the secretory rate (Fig. 6A) after the first 15 minutes following PTHrP injection and over the subsequent 50 to 65 and 65 to 80 minute periods. In both the initial (0 to 35 minutes, before PTHrP injection) and the final (80 to 90 minutes) periods, the mean secretory rates were similar. With hypercalcic medium (Fig. 5B) renin activity rose up to 80 minutes and reached 657 ± 76 Ang I units, which was 1.7-fold higher than in the corresponding time-control IPK. The PTHrP induced increase of the mean secretory rate was significant only during the 35 to 50 minute interval of time, reaching a value of 22.1 ± 1.9 Ang I units/min versus 10.1 ± 1.6 Ang I units/min in the corresponding time-control IPK (Fig. 6B). With perfusate containing 20 nM BAY-K8644, the stimulating action of PTHrP on renin release was even more obvious (Fig. 5C). Renin activity rose up to 80 minutes and reached 489 ± 128 Ang I units, which was about three times higher than in the corresponding time-control IPK. In the same way, PTHrP markedly increased the mean secretory rates after peptide injection up to 80 minutes, whereas no difference was seen during the 0 to 35 minute period and the 80 to 90 minute period (Fig. 6C). Conversely, PTHrP had no effect on the basal renin release enhanced by calcium-free EGTA medium (Figs. 5D and 6D).

Discussion

Evidence for direct renin stimulatory properties of PTHrP

In both single-pass and recirculating constant-flow perfused isolated rat kidneys, the low perfusion pressure and the absence of preexisting vascular tone suppressed the renal vasodilating effect of PTHrP and PTH, as previously described by this laboratory [17, 20] using isolated preconstricted rat kidneys. The perfusion pressures were maintained within a narrow range during the experimental periods to avoid or minimize the effects of hemodynamic changes on renin release. These findings indicate that the baroreceptors would not be responsible for the renin release responses. Under these conditions, the present results are clear evidence that hPTHrP-(1-34) is able to stimulate renin secretion from rat kidney. In addition, the fact that this effect is also displayed by nonfiltering IPK, suggests that the variations in renin release were independent from tubular and macula densa-mediated mechanisms. Taken together, these findings indicate that the variations of renin release to both PTHrP and PTH originated mainly from juxtaglomerular renin-secreting cells.

In the single-pass IPK, PTHrP stimulates renin release with a potency twelve times higher than PTH (EC_{50} = 0.1 nM) and a threshold concentration inducing a significant increase of renin release of 0.01 nM. PTHrP differs from known local renin release promoters, such as isoproterenol, by its short-lasting action (Fig. 3). PTHrP exhibits a number of properties on the renal vasculature: it binds to specific renal arteriolar receptors [16] and stimulates adenylyl cyclase in these vessels [17]; it also vasodilates the renal artery [7] and the isolated rat kidney [17]. Rocacusachs, Dipette and Nickols [6] showed *in vivo* that PTHrP decreases renal vascular resistance without affecting renal blood flow. We now report that PTHrP is, like many renal vasodilators, an effective renin secretion promoter.

This combination of properties is highly suitable for significant modulatory activities of PTHrP on normal renal hemodynamics. Also, a large number of normal human and animal tissues contain immunoreactive PTHrP or express mRNA for PTHrP, suggesting paracrine or autocrine functions for this peptide [8–15]. However, it is still undetermined whether mRNA for PTHrP or PTHrP itself is present or not in the renal vascular smooth muscle cells, but they have been found in a large number of extra-renal vascular tissues. Therefore, a role for PTHrP in the regulation of renal microcirculation is suggested by these findings, but clearly remains unproven. In this respect, the recent findings of Rocacusachs, Dipette and Nickols [6] are of particular interest. In their *in vivo* studies, PTHrP decreased renal vascular resistance without affecting blood flow, while inducing a marked systemic vasodilation and a reduction in mean arterial pressure. In parallel, PTHrP decreased blood flow to the brain and the splanchnic organs, but did not change vascular resistance in these organs. In the light of these results the authors proposed that the reduction in blood pressure most likely resulted in the reflex activation of compensatory pressor systems such as the renin-angiotensin system,

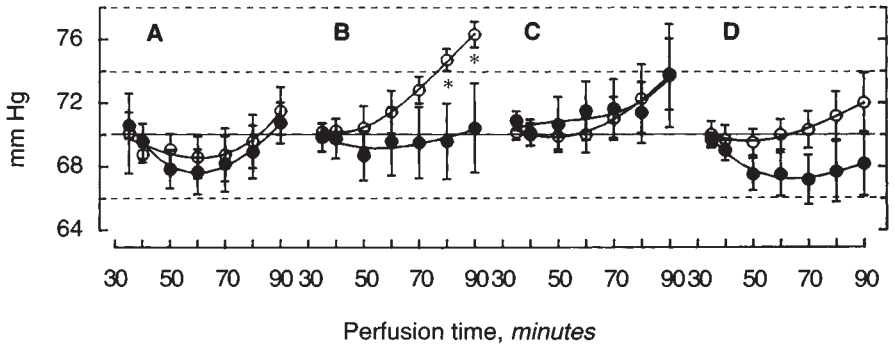

Fig. 4. *Time course of perfusion pressure in the four groups of recirculating IPK.* **(A)** "normocalcic," **B** "hypercalcic," **C** "BAY-K8644 and **D** "EGTA" refer to the experimental conditions described in Table 3. The mean ± SEM are shown. Asterisks indicate significant differences between time-control (○) and PTHrP-treated (●) kidney preparations for a given perfusion time ($P < 0.05$).

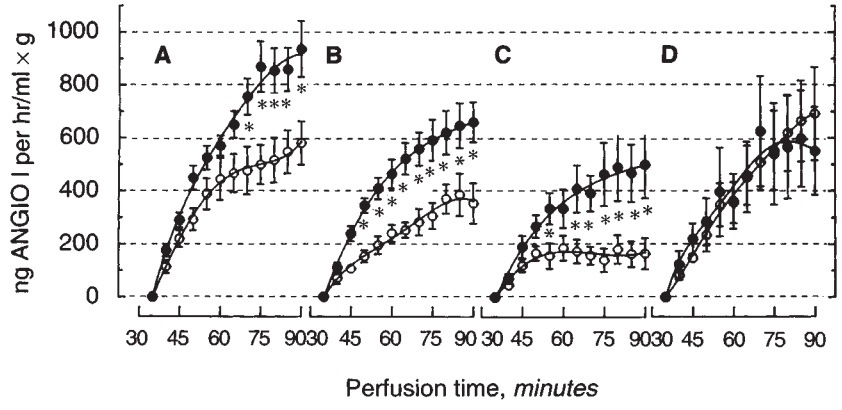

Fig. 5. *Comparison of the time course of the perfusate renin activity concentration between time-control and PTHrP-treated recirculating IPK under various calcium conditions.* IPK were perfused with "normocalcic" **A**, "hypercalcic" **B**, "BAY-K8644" **C** and "EGTA" **D** media as described in Table 3. Results are presented as the net renin activity increase from the end of the initial 35 minute period. The mean levels (± SEM) of perfusate renin activities reached at 35 minutes were 561 ± 70 (A), 414 ± 98 (B), 378 ± 53 (C) and 484 ± 62 Ang I units (D) in time control IPK (○). In PTHrP-treated IPK (●), the 35 minute renin activities were 503 ± 89 (A), 374 ± 89 (B), 378 ± 42 (C) and 482 ± 71 Ang I units (D). Asterisks indicate significant differences between time control and PTHrP-treated IPK for a given perfusion time.

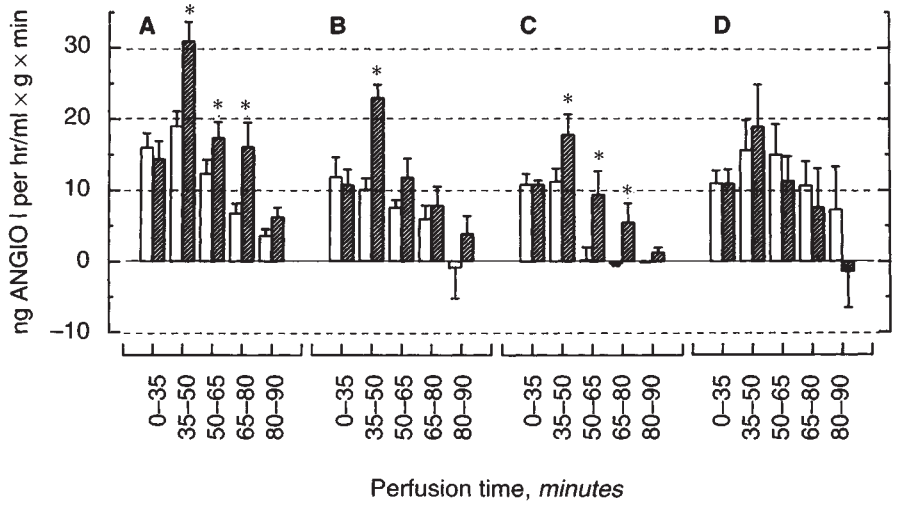

Fig. 6. *Comparison of the time course of the augmentation rate of the perfusate renin activity concentration between time control and PTHrP-treated recirculating IPK perfused under various calcium conditions.* IPK were perfused with "normocalcic" **A**, "hypercalcic" **B**, "BAY-K8644" **C**, and "EGTA" **D** media as described in Table 3. These renin release rates were calculated from the data presented in Figure 5 as described in **Methods**. Asterisks indicate significant differences between time control (□) and PTHrP-treated (▨) IPK for a given perfusion period.

which in turn, might offset the vasodilatory properties of PTHrP in the splanchnic bed. The findings of the present report strongly support such a hypothesis. Thus, it may be of some interest to postulate that, by its action on renin release, PTHrP could contribute to the maintenance of renal blood flow in the face of a reduced systemic pressure. But this assumption clearly needs further investigations.

Insight into the mechanism of the renin stimulating properties of PTHrP

In the time-control recirculating IPK, we observed that renin release increased spontaneously and then reached a maximal level between 50 and 65 minutes and tended to stop thereafter. A similar pattern of renin release accumulation with time was

observed by Cohen et al [29], who showed that hyperoncotic perfusate increased basal renin release from isolated nonfiltering rat kidneys over a period of 70 minutes. In addition, infraphysiologic perfusion pressure used to abolish glomerular filtration may also be involved in the spontaneous increase of renin release during the first 50 to 65 minutes.

The concept that renin secretion is inversely related to extracellular ionized calcium concentration [18, 19, 30, 31] is further documented by the present study. Indeed, increasing the extracellular calcium inhibited renin release, whereas removing extracellular calcium enhanced renin release. Accordingly, BAY-K8644, an analog of the dihydropyridine calcium antagonist nifedipine, which enhances calcium influx through the voltage-dependent channels [32], depressed renin release. PTHrP was unable to further increase basal renin release in absence of extracellular calcium. Conversely, PTHrP strongly reversed the basal renin release depressed by the increase of extracellular calcium or in response to BAY-K8644. These findings suggest that PTHrP stimulates renin release from IPK through a calcium-dependent process. At this point, the question of whether PTHrP inhibits calcium entry in renin secreting cells as it has been suggested previously for PTH in vascular smooth muscle cells [33–35], requires direct measurements of cytosolic calcium. However, another possibility is that basal and stimulated renin releases are derived from different intracellular pools controlled by different mechanisms. Thus, it is possible that basal renin release is derived from one pool which is inhibited by rises in intracellular calcium. A second pool may be coupled to local rises in intracellular calcium and be dependent on extracellular calcium. As PTHrP stimulates adenylyl cyclase activity in renal renin containing arterioles [17], the possibility exists that PTHrP promotes renin release by a process depending on both cAMP generation and extracellular calcium. In the present work, we also demonstrate that the products of cyclooxygenase activity do not account for the stimulatory properties of PTHrP on renin release. In other respects, the lack of additivity of the renin release responses to PTHrP and PTH suggests that these peptides stimulate renin release by interaction with common action sites. Further investigations such as binding studies are needed to assess this conclusion.

In summary, the data presented here support a stimulatory role for PTHrP on renin release. This effect is dependent on extracellular calcium and independent on baroreceptors, macula densa and prostaglandin synthesis. Finally, PTHrP action on renin release most likely results from activation of renal vascular PTH action sites.

Acknowledgments

This work was supported by the French National Institute of Health (INSERM) Grants CRE897005 and CRE920203, the Association of Cancer Research (ARC) and La Direction de la Recherche et des Etudes Doctorales, MENC, France (recipient JJH). We also wish to thank FRESENIUS Gmbh, Germany for financial contribution. We thank Professors C. Bollack, J. Geisert and T. Hannedouche (Hopitaux Universitaires de Strasbourg) for advice and encouragement and Dr M.J. Musso (Lilly France, Strasbourg) for stimulating discussions. We also thank J. Krill, S. Wendling and S. Rothhut for their technical assistance.

Reprint requests to Jean-Jacques Helwig, Ph.D., Laboratoire de Physiologie Cellulaire Rénale, Pavillon Poincaré, Hopitaux Universitaires, B.P. 426, 67091 Strasbourg Cedex, France.

References

1. Halloran BP, Nissenson RA: Parathyroid Hormone-related Protein, Boca Raton, CRC Press, 1993
2. Martin TJ: Properties of parathyroid hormone-related protein and its role in malignant hypercalcemia. *Q J Med* 76:771–786, 1990
3. Stewart AF, Broadus AE: Parathyroid hormone-related protein. *J Clin Endocrinol Metab* 71:1410–1414, 1990
4. Stewart AF, Broadus AE: Humoral hypercalcemia of malignancy, in *Advances in Endocrinology and Metabolism*, edited by Mazzaferri E, Chicago, Year Book Medical Publishers, 1990, pp. 1–21
5. Nickols GA, Nana AD, Nickols MA, Dipette DJ, Asimakis GK: Hypotension and cardiac stimulation due to the parathyroid hormone-related protein, humoral hypercalcemia of malignancy factor. *Endocrinology* 125:834–841, 1989
6. Rocacusachs A, Dipette DJ, Nickols GA: Regional and systemic hemodynamic effects of parathyroid hormone-related protein—Preservation of cardiac function and coronary and renal flow with reduced blood pressure. *J Pharmacol Exp Ther* 256:110–118, 1991
7. Winquist RJ, Baskin EP, Vlasuk GP: Synthetic tumor derived human hypercalcemic factor exhibits parathyroid hormone-like vasorelaxation in renal arteries. *Biochem Biophys Res Commun* 149:227–232, 1987
8. Rubin LP, Posillico JT, Anast CS, Brown EM: Circulating levels of biologically active and immunoreactive intact parathyroid hormone in human newborns. *Pediatr Res* 29:201–207, 1991
9. Kramer S, Reynolds FH, Castillo M, Valenzuela DM, Thorikay M, Sorvillo JM: Immunological identification and distribution of parathyroid hormone-like protein polypeptides in normal and malignant tissues. *Endocrinology* 128:1927–1937, 1991
10. Selvanayagam P, Graves K, Cooper C, Rajaraman S: Expression of the parathyroid hormone-related peptide gene in rat tissues. *Lab Invest* 64:713–717, 1991
11. Thiede MA, Daifotis AG, Weir EC, Brines ML, Burtis WJ, Ikeda K, Dreyer BE, Garfield RE, Broadus AE: Intrauterine occupancy controls expression of the parathyroid hormone-related peptide gene in preterm rat myometrium. *Proc Natl Acad Sci USA* 87:6969–6973, 1990
12. Thiede MA, Harm SC, Hasson DM, Gardner RM: *In vivo* regulation of parathyroid hormone-related peptide messenger ribonucleic acid in the rat uterus by 17beta-estradiol. *Endocrinology* 128:2317–2323, 1991
13. Moseley JM, Hayman JA, Danks JA, Alcorn D, Grill V, Southby J, Horton MA: Immunohistochemical detection of parathyroid hormone-related protein in human fetal epithelia. *J Clin Endocrinol Metabol* 73:478–484, 1991
14. Thiede MA, Harm SC, Mckee RL, Grasser WA, Duong LT, Leach RM: Expression of the parathyroid hormone-related protein gene in the avian oviduct—Potential role as a local modulator of vascular smooth muscle tension and shell gland motility during the egg-laying cycle. *Endocrinology* 129:1958–1966, 1991
15. Hongo T, Kupfer J, Enomoto H, Sharifi B, Giannellaneto D, Forrester JS, Singer FR, Goltzman D, Hendy GN, Pirola C, Fagin JA, Clemens TL: Abundant expression of parathyroid hormone-related protein in primary rat aortic smooth muscle cells accompanies serum-induced proliferation. *J Clin Invest* 88:1841–1847, 1991
16. Nickols GA, Nickols MA, Helwig JJ: Binding of parathyroid hormone and parathyroid hormone-related protein to vascular smooth muscle of rabbit renal microvessels. *Endocrinology* 126: 721–727, 1990
17. Musso MJ, Plante M, Judes C, Barthelmebs M, Helwig JJ: Renal vasodilation and microvessel adenylate cyclase stimulation by synthetic parathyroid hormone-like protein fragments. *Eur J Pharmacol* 174:139–151, 1989
18. Fray JCS, Park CS, Valentine AND: Calcium and the control of renin secretion. *Endocrine Rev* 8:53–93, 1987
19. Hackenthal E, Paul M, Ganten D, Taugner R: Morphology, physiology, and molecular biology of renin secretion. *Physiol Rev* 70:1067–1116, 1990

20. Musso MJ, Barthelmebs M, Imbs JL, Plante M, Bollack C, Helwig JJ: The vasodilator action of parathyroid hormone fragments on isolated perfused rat kidney. *Naunyn Schmiedebergs Arch Pharmacol* 340:246–251, 1989
21. Helwig JJ, Musso MJ, Judes C, Nickols GA: PTH and calcium: Interactions in the control of renin secretion in the isolated nonfiltering rat kidney. *Endocrinology* 129:1233–1242, 1991
22. Saussine C, Judes C, Massfelder T, Musso MJ, Simeoni U, Hannedouche T, Helwig JJ: Stimulatory action of parathyroid hormone on renin secretion *in vitro*: A study using isolated rat kidney, isolated rabbit glomeruli and superfused dispersed rat juxtaglomerular cells. *Clin Sci* 84:11–19, 1993
23. Schurek HJ, Brecht JPH, Hierholzer K: The basic requirements for the function of the isolated cell free perfused rat kidney. *Pflügers Arch* 354:349–365, 1975
24. Maack T: Physiological evaluation of the isolated perfused rat kidney. *Am J Physiol* 238:F71–F78, 1980
25. Itoh S, Carretero OA, Murray RD: Renin release from isolated afferent arterioles. *Kidney Int* 27:762–767, 1985
26. Fyhrquist F, Soveri P, Puutula L, Stenman UH: Radioimmunoassay of plasma renin activity. *Clin Chem* 22:250–256, 1976
27. Winer BJ: Design and analysis of factorial experiments, in *Statistical Principles in Experimental Design*, New York, McGraw Hill, 1971
28. Duncan DB: Multiple range and multiple F tests. *Biometrics* 11:1–42, 1955
29. Cohen AJ, Spokes K, Brown RS, Stoff JS, Silva P: Stimulation of renin release by hyperoncotic perfusion of the isolated rat kidney. *Circ Res* 50:400–404, 1982
30. Fray JCS: Control of Renin Secretion by Extracellular Calcium. *Cell Calcium* 11:339–341, 1990
31. Pardy K, Williams BC, Noble AR: Inhibitory role of $Ca2^+$ in the control of renin secretion: A study using superfused dispersed rat renal cortical cells. *Clin Sci* 77:273–279, 1989
32. Yamamoto H, Huang O, Vanbreemen C: BAY-K8644 differentiates between potential and receptor operated calcium channels. *Eur J Pharmacol* 102:555–563, 1984
33. Pang PKT, Yang MCM, Sham JSK: Parathyroid hormone and calcium entry blockade in a vascular tissue. *Life Sci* 42:1395–1400, 1988
34. Pang PKT, Wang R, Shan J, Karpinski E, Benishin CG: Specific inhibition of long-lasting, L-type calcium channels by synthetic parathyroid hormone. *Proc Natl Acad Sci USA* 87:623–627, 1990
35. Wang R, Karpinski E, Pang PKT: Parathyroid hormone selectively inhibits L-type calcium channels in single vascular smooth muscle cells of the rat. *J Physiol (Lond)* 441:325–346, 1991